Mechanisms Underlying Nitroglycerin-Induced Superoxide Production in Platelets

Some Insight, More Questions

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Nitroglycerin (NTG) has been the foremost anti-ischemic agent used in clinical medicine for more than a century. NTG is a potent vasodilator of veins, arterial conductance vessels, and collaterals that has minimal effects on arteriolar tone. At the cellular level, NTG is biotransformed by a still unknown enzymatic process in endothelial cells, smooth muscle, and to some extent platelets, causing it to release the vasodilator and anti-aggregatory principle nitric oxide. The 2 major drawbacks of nitrate therapy that have been shown to be important are the rapid development of nitrate tolerance and endothelial dysfunction within several days of prolonged NTG treatment. There is a growing body of evidence that both NTG-induced side effects may be at least in part secondary to NTG-stimulated production of oxygen-derived free radicals within vascular tissue. In increased oxidative stress correlates positively with increased cardiovascular event rates, which may, at least in part, explain why NTG-therapy either failed to improve or worsened the prognosis in patients with acute myocardial infarction and chronic ischemic heart disease, respectively.

The weak antiplatelet activity of NTG, such as that in washed platelet preparations, may indicate that, in contrast to NO-donors (eg, sodium nitroprusside) that release NO spontaneously, the biotransformation capacity of platelets to form NO from NTG is clearly limited. In contrast, the addition of plasma or endothelial cells and/or smooth muscle cells strikingly increases NTG-induced platelet inhibition, indicating that by providing the necessary metabolic activity to form NO from NTG, the anti-aggregatory effects of NTG may be of clinical relevance. The mechanisms by which NTG and NO inhibit platelet aggregation include the stimulation of the soluble guanylyl cyclase, leading to enhanced formation of the second messenger cGMP, but may also involve cGMP-independent mechanisms.

More recent studies indicate that platelet aggregation is also regulated through NO-derived from constitutive nitric oxide synthase (eNOS). NO release has been reported to occur in resting and aggregating platelets. Interestingly, platelets also produce superoxide during aggregation. Superoxide and NO may react readily to produce peroxynitrite. It is therefore possible that platelet superoxide production modifies the bioavailability of NO for platelets. For this reason, it is relevant to always define both NO and superoxide release from platelets.

In this issue of Circulation, McVeigh et al present a paper that examines the effects of long-term nitrate therapy on platelet NO and superoxide production, and how vitamin C affects these phenomena. As an additional parameter of intracellular NO-bioavailability, platelet cGMP content was determined. Platelet superoxide was measured in response to stimulation with the phorbolester PMA (a direct activator of protein kinase C), whereas platelet NO release was determined after activation with ADP. The authors also looked at the development of hemodynamic tolerance by performing radial artery pressure analysis as described previously. The study design was a placebo-controlled crossover trial with random allocation of therapies.

Treatment of healthy volunteers with a relatively low dose of NTG (NTG-patch, release rate 0.4 mg/h; ≈0.07 μg · kg⁻¹ · min⁻¹) for 3 consecutive days increased platelet NO production 2.5-fold and superoxide production 4.5-fold. Surprisingly, the excess increase in superoxide was associated with a sustained increase in platelet cGMP and significant hemodynamic effects. The NTG-induced increases in platelet NO and superoxide production were inhibited by concomitant therapy with vitamin C.

From where is the superoxide coming? As discussed by McVeigh et al., a significant contribution of superoxide producing enzymes, such as the xanthine oxidase, mitochondrial NADH dehydrogenase, and cyclooxygenase, was ex-
cluded because specific inhibitors of these enzymes, namely oxypurinol, rotenone, and indomethacin, respectively, did not modify platelet superoxide production. The observed simultaneous increase in platelet NO and superoxide release may indicate the formation of peroxynitrite, which may cause NOS uncoupling in platelets due to oxidation of the NOS cofactor tetrahydrobiopterin.\textsuperscript{14,15} To assess the potential contribution of NOS to superoxide production, the authors used the NOS inhibitor L-NAME.\textsuperscript{11} In all groups studied, however, L-NAME increased rather than decreased superoxide production. Thus, unlike observations in vascular tissue,\textsuperscript{16} uncoupled cNOS does not seem to contribute significantly to the observed increase in platelet superoxide.

The next potential superoxide-producing enzyme the authors studied was NAD(P)H oxidase. In endothelial, smooth muscle, and adventitial cells, this enzyme has been shown to contribute considerably to vascular superoxide production in various disease states, such as hypertension and atherosclerosis,\textsuperscript{17} but it has also been shown to increased vascular superoxide in nitrate tolerance.\textsuperscript{18} Previous studies\textsuperscript{19} have indicated that the NAD(P)H cytochrome c reductase enzyme, which catalyzes superoxide anion production, is present in platelets with high specific activity. More recent studies\textsuperscript{20} point to the existence of this enzyme by showing the expression of the NAD(P)H oxidase subunits p22phox and p67phox in platelets and their precursors, the megakaryocytic cells. In these studies, the activity of the enzyme was assessed with not only lucigenin-enhanced chemiluminescence but also with electron spin resonance using 5,5-dimethyl-1-pyrroline-N-oxide as a spin trap. Although exposure to phorbolester greatly enhanced the activity of the enzyme in platelets in response to NADH and NAD(P)H, a complete inhibition was observed in response to diphenylene iodonium (DPI).\textsuperscript{20}

To assess the contribution of the NADH/NAD(P)H oxidase in platelets from NTG-treated subjects, McVeigh et al\textsuperscript{11} stimulated homogenized platelets with NTG and NAD(P)H. In platelets from NTG-treated subjects, the NADH-driven superoxide production was significantly increased, suggesting that an enzyme that uses this as a substrate must be activated or upregulated in platelets as a result of NTG treatment. Further evidence for the involvement of this oxidase was provided because 2 inhibitors, DPI and quinacrine, completely abolished superoxide production in platelets and also significantly reduced the NADH-induced superoxide formation.

In the present study,\textsuperscript{11} the authors also measured NO-release in response to platelet aggregation. NTG-treatment for 3 days lead to a 2.5-fold increase in NO release as assessed with an NO-sensitive electrode and by the determination of nitrite levels in the supernatant. Because eNOS activity was not changed, it is unlikely that platelet NOS contributed significantly to the increase in NO release. Increased NO-release could also be derived from NO-storage molecules formed in response to NTG-treatment, including nitrated/nitrosated platelet proteins\textsuperscript{22} or nitrolipids,\textsuperscript{23} which may release NO on aggregation of platelets. It is important to stress that nitrination of specific surface proteins of platelets may also account for cGMP-independent inhibition of platelet aggregation.

As an additional marker for intracellular NO bioavailability in platelets, cGMP levels were determined. Surprisingly, in response to this relatively low concentration of NTG, a small but significant increase in cGMP was observed and was therefore not subject to "tolerance."\textsuperscript{11} Nevertheless, as pointed out by the authors, the issue of whether NTG therapy can increase platelet cGMP content is unclear, and there is a consensus that measurement of platelet cGMP levels cannot be recommended as a biochemical marker for nitrate tolerance development.

To assess hemodynamic tolerance, radial artery pulse wave analysis was performed. After 3 days of NTG treatment, a significant effect of NTG on pulse wave morphology that did not significantly differ from patients treated with the combination of NTG and vitamin C was still apparent.\textsuperscript{11} Because the authors did not measure these parameters immediately after initiation of NTG treatment or on day 1 and/or on day 2, one cannot exclude the possibility that some degree of tolerance had developed, as shown previously using the same method.\textsuperscript{13}

The present study also indicates that concomitant treatment with vitamin C was able to inhibit NTG-induced stimulation of superoxide production in platelets from NTG-treated subjects. This report fits with previous observations, where vitamin C was able to prevent the development of hemodynamic tolerance in patients with congestive heart failure\textsuperscript{24} and in healthy volunteers,\textsuperscript{17} as well as to restore the activity of the cGMP-dependent protein kinase in vascular tissue.\textsuperscript{25} Because the prevention of tolerance by vitamin C has been shown to be associated with a reduction in vascular superoxide levels,\textsuperscript{25} it is conceivable that the inhibitory effects of vitamin C on platelet-superoxide production observed in the present study is due to free radical scavenging effects. A surprising observation was the simultaneous reduction in platelet NO-release. By reducing platelet superoxide levels, platelet NO bioavailability, and therefore platelet NO and nitrite levels, should increase rather than decrease. Interestingly, recent studies\textsuperscript{22} have shown that vitamin C inhibits the degree of nitrosation reactions of cellular proteins in response to NO. It is therefore tempting to speculate that a similar phenomenon accounts for the observed paradoxical decrease in NO release in aggregation of platelets from NTG/vitamin C-treated subjects.

Although the authors detected an increase in platelet derived-superoxide, no information concerning functional consequences was provided. Incubation of platelets with DPI has been shown to inhibit platelet aggregation.\textsuperscript{26} Because DPI inhibits flavin-dependent oxidoreductases and therefore both platelet NOS and NAD(P)H oxidase, this observation may indicate that basal superoxide production may already decisively modulate platelet function. Future studies will be needed to answer the open question of whether the observed stimulatory effect of NTG on platelet superoxide adversely affects the aggregatory characteristics of platelets. Further questions include whether NTG-therapy stimulates the formation of platelet esterified 8-epi PGF\textsubscript{2alpha}, a specific marker of lipid peroxidation, and whether the simultaneous increase in platelet NO and superoxide levels leads to increased forma-
tion of peroxynitrite. Are there changes in the expression of the platelet NAD(P)H oxidase subunits p22phox and p67phox in response to NTG treatment, and what are the precise mechanisms underlying the activation of this enzyme? It will also be important to determine whether platelet superoxide levels are reduced by concomitant treatment with ACE inhibitors or AT1 receptor antagonist and whether the mitochondrial aldehyde dehydrogenase is involved in NTG-mediated increases in platelet superoxide.26

The results from the study by McVeigh et al11 demonstrate for the first time quite a marked stimulatory effect of NTG on platelet superoxide production, which seems to be, at least in part, secondary to activation of the platelet NAD(P)H oxidase. Interestingly, several studies have linked the phenomenon of “nitrate resistance” in platelets to the anti-aggregatory actions of NTG, with increased platelet superoxide levels occurring in patients with diabetes, congestive heart failure,27 unstable angina, and myocardial infarction.28 Therefore, it is tempting to speculate that NTG-induced superoxide production is partly responsible for “nitrate tolerance” in platelets, as demonstrated by the results from previous clinical trials.29,30 We agree that platelets exposed to clinically relevant concentrations of NTG may represent a convenient compartmentalized tissue model that permits the study of NO/superoxide interactions that have been thought to play a role in nitrate tolerance.

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References
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